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Formation of alanine, α-aminobutyrate, acetate, and 2-butanol during cheese ripening by *Pediococcus acidilactici* FAM18098

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ABSTRACT

There is limited information about the contribution of *Pediococcus acidilactici*, a nonstarter lactic acid bacteria, to cheese ripening and flavour development. Model Tilsit-type and Gruyère-type cheeses were produced using *P. acidilactici* FAM18098 as an adjunct. The adjunct did not influence the cheese manufacturing processes. The pediococcal log counts ranged from 7.0 to 8.0 cfu g^{-1} after 90 and 120 days of ripening. *P. acidilactici* produced ornithine, a result of arginine metabolism by the arginine deiminase pathway, and α-aminobutyrate and alanine while simultaneously metabolising serine and threonine. The analysis of the volatile compounds in the cheeses showed that higher acetate, 2-butanone, and 2-butanol levels and lower diacetyl levels were present in the cheeses produced with *P. acidilactici* than in the control cheeses. The study illustrates that *P. acidilactici* can influence amino acid metabolism in cheese; further, ornithine, α-aminobutyrate, and acetate can serve as indicators for the presence of this species.

1. Introduction

During cheese ripening, proteases and peptidase degrade the caseins to peptides and amino acids. Determining free amino acids in cheese using high-performance liquid chromatography (HPLC) is an important method for studying this process. However, not only amino acids are released from the caseins: nonproteinogenic amino acids such as α-aminobutyrate (AABA, 2-aminobutyrate), γ-aminobutyrate (GABA, 4-aminobutyrate), citrulline, and ornithine are also liberated; these result from microbial activity and can also be analysed (Bütikofer & Ardö, 1999). Citrulline and ornithine are products of the arginine deiminase pathway: arginine is deaminated by the activity of arginine deiminase (EC 3.5.3.6) to produce citrulline and ammonia, subsequently ornithine carbamoyltransferase (EC 2.1.3.3) converts citrulline to ornithine and carbamoyl phosphate. The decarboxylation of glutamate produces GABA, glutamate decarboxylases (EC 4.1.1.15) catalysing the reaction. Knowledge about microbial AABA biosynthesis is limited, but there are indications that it is derived from threonine (Fotheringham, Grinter, Pantaleone, Senkpeil, & Taylor, 1999; Tao, Jiang, Zhu, & Yang, 2014).

Agroscope in Liebefeld, Switzerland has performed analyses of free amino acids in cheese for many years; the research institution has repeatedly detected AABA without knowing which microorganisms were involved in its formation. Later, Irmler et al. (2013) found that the dairy isolate *Pediococcus acidilactici* FAM18098 was capable of producing this compound. Further studies (Irmler, unpublished data) showed that this phenotype was detectable in various strains of this species.

P. acidilactici is a homofermentative Gram-positive lactic acid bacterium that produces D- and L-lactate from carbohydrates (Holzapfel, Franz, Ludwig, Back, & Dicks, 2006). Cocci, which form tetrads, can be observed on the microscopic level. The bacterium is occasionally found in the bacterial nonstarter populations of raw milk cheeses at the end of the ripening process. It has been isolated from a range of globally manufactured cheese varieties, such as Caciocavallo Palermitano (Guarrasi et al., 2017), feta and kasseri cheese (Litopoulou-Tzanetaki, Vafopoulou-Mastrojiannaki, & Tzanetakis, 1989), Montasio cheese (Carraro et al., 2011), Parmigiano Reggiano cheese (Coppola et al., 1997), Columbian double cream cheese (Londoño-Zapata, Durango-Zuleta, Sepúlveda-Valencia, & Moreno Herrera, 2017), raw goats' milk cheeses (Picon, Garde, Ávila,

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& Nuñez, 2016), and various raw milk cheeses traditionally produced in Switzerland, such as Le Gruyère, Emmental, Appenzeller, and Tilsit (Isolini & Fröhlich-Wyder, 2003).

There are several previous studies that have examined the physiological and biochemical properties of the closely related *Pediococcus pentosaceus*. These studies mainly focused on the proteolytic and lipolytic activities of this species (Bhowmik & Marth, 1989, 1990; Litopoulou-Tzanetaki et al., 1989; Tzanetakis & Litopoulou-Tzanetaki, 1989). Additionally, researchers determined that *P. pentosaceus* produced acetate from lactate in the presence of oxygen (Thomas, McKay, & Morris, 1985). In low-fat Cheddar cheese produced with a *P. pentosaceus* strain, enhanced proteolysis and higher levels of acetate were detected than in the control cheeses produced without this strain (Bhowmik, Riesterer, van Boekel, & Marth, 1990). Vafopoulou-Mastrojiannaki, Litopoulou-Tzanetaki, and Tzanetakis (1990) reported that the presence of *P. pentosaceus* enhanced proteolysis and shortened the maturation time of feta cheese by one month.

Less knowledge about *P. acidilactici* is available. Several researchers reported that strains of this species did not exhibit esterolytic, lipolytic, or proteolytic activity (Bhowmik & Marth, 1989; Litopoulou-Tzanetaki et al., 1989). Other researchers used cell-free extracts, finding protease and peptidase activity (Bhowmik & Marth, 1990).

The aim of the present research was to study the influence of *P. acidilactici* on proteolysis, lipolysis, lactate metabolism, and the formation of volatile organic compounds (VOCs) in model-Tilsit cheese and model-Gruyère cheese. Its capability to metabolise amino acids was analysed using HPLC.

2. Materials and methods

2.1. Cultures, biochemical properties, and physiological properties

The *P. acidilactici* strain FAM18098, which was isolated from Swiss Gruyère protected designation of origin (PDO) cheese, was grown in de Man, Rogosa, and Sharpe (MRS) broth (de Man, Rogosa, & Sharpe, 1960) at 30 °C. The species of the strain FAM18098 was determined by sequencing part of the *tuf* gene using the degenerated primers described by Ke et al. (1999). For long-term storage, the pediococcal strain was stored in a 2% (v/w) sterile skim milk powder solution at −80 °C.

The mixed starter cultures MK 401, RMK 101, and RMK 124 (Agroscope, Liebefeld, Switzerland) were used for the cheese making (www.liebefeld-kulturen.ch). MK 401 is composed of an undefined mixture of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *lactis*, and *Lactococcus lactis* subsp. *lactis*. RMK 101 and RMK 124 contain an undefined mixture of *S. thermophilus* and *Lb. delbrueckii* subsp. *lactis*.

The degradation of arginine and the formation of ornithine were assayed using an arginine-containing medium (MAM) and high-performance thin-layer chromatography (HPTLC), as described previously (Wenzel et al., 2018). The formation of alanine and α-aminobutyrate was assayed in a broth consisting of potassium phosphate (9 g L^{-1}), yeast extract (5 g L^{-1}), casein hydrolysate (1 g L^{-1}), magnesium sulphate (0.1 g L^{-1}), manganese sulphate (0.1 g L^{-1}), D-galactose (2 g L^{-1}), L-serine (0.5 g L^{-1}), and L-threonine (0.6 g L^{-1}) using thin-layer chromatography (TLC), as described previously (Irmler et al., 2013). To study the capability to ferment citrate, cultures were plated on agar plates containing insoluble calcium citrate (Galesloot, Hassing, & Stadhouders, 1961). The medium used in this study was prepared as follows: 600 mL of a medium containing 3.0 g of calcium L-lactate, 9 g of proteose peptone, 6.0 g of tryptone, 6.0 g of yeast extract, 1.8 g of D-glucose, and 0.6 g of Tween-80 and a suspension of 40 mL of 0.6 g of carboxyl-methyl-cellulose and 4 g of calcium citrate; both solutions were autoclaved separately, mixed, and poured into petri dishes. Inoculated plates were incubated at 30 °C and 37 °C under anaerobic conditions. The consumption of citrate can be observed by the development of clear zones around the bacterial colonies.

2.2. Cheese production

The study comprises four model Tilsit-type (semi-hard) cheeses that were ripened for 90 days, four model Tilsit-type cheeses that were ripened for 120 days, and four model Gruyère-type (hard cheeses) that were ripened for 120 days. A control cheese and a corresponding cheese with the pediococcal adjunct were produced on six different days at the Agroscope pilot plant (Liebefeld, Switzerland). A different milk batch was used for each production day.

The model Tilsit-type (semi-hard) cheeses were produced from 70 L of pasteurised full-fat (37 g kg^{-1}) cows' milk. The acidification was started by adding 0.2% of the starter culture MK401. Additionally, 10% of water was added to slow down the acidification rate. The pediococcal adjunct culture, which had been grown in the MRS broth, was added at a final population density of 3–4 log colony-forming units (cfu) per mL of vat milk together with the starter culture. The milk was incubated at 31–32 °C for 15 min. Then, 10 mL of Winkler GR orange rennet (Winkler AG, Konolfingen, Switzerland) with 194 international milk-clotting units mL^{-1} diluted in 1 L of water was added to the vat of milk. After undergoing an additional incubation at 32 °C for 30 min, the coagulum was cut into cubes of approximately 10 mm each. Subsequent to the addition of 20 L of water, the curds and whey mixture was cooked at 44 °C for 20 min followed by additional stirring (43 °C for 20 min). The curds were then transferred into perforated moulds and pressed for 7.5 h. The cheeses were immersed in a brine solution for 16 h at 11–13 °C.

The model Gruyère-type (hard) cheeses were produced from 120 L of pasteurised full-fat cows' milk. The acidification was initiated by adding 0.1% of the starter culture RMK 101 and 0.1% of the starter culture RMK 124. *P. acidilactici* was added at a final population density of 3–4 log cfu mL^{-1} of vat milk together with the starter culture. The milk was incubated at 31–32 °C for 30 min. Then, 13 mL Winkler GR orange rennet was diluted in 1 L of water and added to the vat of milk. After undergoing additional incubation at 32 °C for 40 min, the coagulum was cut into cubes of approximately 4 mm each. The curds and whey mixture was cooked at 56 °C for 30 min followed by additional stirring (56 °C for 20 min). The curds were then transferred into perforated moulds and pressed for 20 h; then, the cheese was immersed in a brine solution for 24 h.

An influence of the pediococcal adjunct on the acidification rate was not observed. All the cheeses were finally ripened at 14–15 °C with 90–96% relative humidity. Cheese smearing was performed daily using a brine solution that was inoculated with a mixture of *Brevibacterium linens*, *Arthrobacter* spp., and *Debaryomyces hansenii* (OMK 702; Agroscope, Liebefeld, Switzerland). After 10 days, the brine solution was applied twice per week.

2.3. Cheese sampling

Samples were taken aseptically from the cheeses after 90 or 120 days of ripening. The rind (5 mm thick) was discarded, and the remaining cheese sample was ground up and mixed for analysis.

2.4. Chemical analyses

L-Lactate, D-lactate, and citrate values were determined using enzymatic methods with commercially available kits (R-Biopharm AG, Darmstadt, Germany). The total nitrogen (TN), water-soluble nitrogen (WSN), and 12% trichloroacetic acid-soluble nitrogen (TCA-N) were analysed as described by Collomb, Spahni, and Steiger (1990). Fat content was measured according to the International Dairy Federation's methods (IDF, 2008), and water content was determined by drying to a constant weight (IDF, 2004). Free amino acids were determined using HPLC (Bütikofer & Ardö, 1999); short-chain fatty acid (SCFA) levels were determined using gas chromatography (GC) as described previously (Fröhlich-Wyder et al., 2013).

2.5. Estimation of the pediococcal population density

Ten grams of each cheese sample were homogenised in 90 mL of 40 °C warm peptone water (10 g L^{-1} peptone from casein, 5 g L^{-1} sodium chloride, 20 g L^{-1} trisodium citrate dihydrate, pH 7.0) using a stomacher (Masticator; IUL Instruments GmbH, Königswinter, Germany). Serial decimal dilutions of the suspensions were plated on MRS agar plates that were supplemented with 1 μg mL^{-1} of ampicillin and incubated at 30 °C for 48 h under anaerobic conditions.

2.6. Headspace solid phase microextraction and gas chromatography

The volatile organic compounds (VOCs) were analysed using dynamic headspace (HS) sampling with solid phase microextraction (SPME) followed by the separation and detection of the compounds using GC and mass spectrometry (MS), respectively. For sample preparation, 2 cm of the rind and cheese were removed from all sides of the cheese to provide samples that were as homogeneous as possible. The cut cheese samples (3.00 ± 0.10 g) were placed in 20 mL HS vials (Interchim, Montluçon, France), then mixed with 6 mL 0.2 mol L^{-1} phosphate buffer solution, pH 7.6. The cheese suspension was homogenised with 10 μL of an internal standard solution containing paraldehyde (200 mg kg^{-1} in deionised water) and the buffer solution. Two replicates were prepared for each sample, and the samples were stored at 4 °C until the analysis.

For SPME, a 2 cm divinylbenzene/carboxen/polydimethylsiloxane 50/30 μm StableFlex fibre (Supelco Inc., Bellefonte, PA, USA) was used to extract the organic volatiles. The fibre was conditioned according to the supplier's recommendations (270 °C for 60 min). The analyses were conducted using a multipurpose auto sampler equipped with Maestro 1 software version 1.4.8.14/3.5 (MPS2; Gerstel AG, Sursee, Switzerland) and a 7890B GC system coupled with a 5977A mass selective detector (Agilent Technologies, Santa Clara, CA, USA).

The HS was incubated for 10 min at 45 °C; then, it was extracted for 45 min at 45 °C with an agitation rate of 250 rpm. The bound volatiles were desorbed for 1 min at 250 °C in the injector, which was in the splitless mode for 30 s; then, the split valve was opened (split flow: 80 mL min^{-1}). The volatile compounds were separated using an HP-5ms Ultra Inert fused silica capillary column (30 m × 0.25 mm, 0.25 μm film; Agilent Technologies, Santa Clara, CA, USA), with helium as the carrier gas at a constant flow of 2.1 mL min^{-1} (37 cm s^{-1}).

The oven temperature was programmed as follows: 3 min at 40 °C, then heated to 140 °C at a rate of 5 °C min^{-1}, and then heated to 250 °C at a rate of 8 °C min^{-1}; the final hold time was 10 min. The MS was set at 230 °C for the transfer line and source temperature, and the analytes were monitored in the scan mode between 30 and 150 amu without a solvent delay.

The detectors response signals were integrated using ChemStation data analysis software version E.02.00.493 (Agilent Technologies, Santa Clara, CA, USA). The National Institute of Standards and Technology (NIST)/United States Environmental Protection Agency/National Institutes of Health Mass Spectral Database (NIST 11) version 2.0 (NIST, Gaithersburg, MD, USA) was used for peak identification.

2.7. Experimental design and statistical analysis

The experimental design included controls and variants made with *P. acidilactici* FAM18098 as an adjunct. The statistical analysis was carried out on the instrumental data with an analysis of variance and a general linear model using SYSTAT V12 software (Systat Software, Inc., San Jose, CA, USA). For this study, the significant differences between the various factors were reported at $P < 0.05$. The factor "adjunct," cheese type, and the interaction adjunct × cheese type were treated as categorical variables. For the statistical analysis of the VOCs, the values (total ion counts) obtained from the cheeses with the adjunct were standardised to the values of the corresponding control cheeses. This was not done for the compound 2-butanol, as the control cheeses did not contain this substance.

3. Results and discussion

3.1. Pediococcal counts

To the present researchers' knowledge, a selective medium to enumerate pediococci has not yet been described. This study used a medium that had been successfully applied for the isolation of pediococcal species from cheese (Isolini & Fröhlich-Wyder, 2003) and only enumerated pediococci in the semi-hard cheeses. For the cheeses made with *P. acidilactici* FAM 18098 and ripened for 90 days, population densities of 7.9 (±0.02) log cfu g^{-1} cheese were counted. In the two corresponding control cheeses ripened for 90 days, no colonies developed from one cheese, whereas the other cheese exhibited 5 log cfu g^{-1}. This suggests that an ampicillin-resistant bacterium originating from the raw milk, the equipment or the pilot plant environment survived the pasteurisation process and grew while the cheese was ripening. Because the chemical data from this control cheese did not reveal considerable differences to the second control cheeses, this study argues that the presence of this bacterium (which was not characterised any further) had no influence on the overall outcomes of the study; therefore, the results from this control cheese were included in the further analyses.

The batches of semi-hard cheeses that were ripened for 120 days revealed population densities of log 7.4 (±0.2) cfu g^{-1} for *P. acidilactici*. No colonies were found in the corresponding control cheeses.

The population densities of pediococci in cheese are sparsely found in the literature, which is probably because a selective medium for this species does not exist. Isolini and Fröhlich-Wyder (2003) reported that *P. acidilactici* reached population densities of approximately log 6.5 cfu g^{-1} in Tilsit cheese after 49 days of ripening. The closely related *P. pentosaceus* was found to reach population densities of 7 log cfu g^{-1} in low-fat Cheddar cheese after three months of ripening and 8.2 log cfu g^{-1} of the feta cheese after 45 days of ripening (Bhowmik et al., 1990; Vafopoulou-Mastrojiannaki et al., 1990). It is well known that for several cheese types, the adventitious mesophilic lactobacilli, which are closely related to pediococci, increase during ripening from approximately log 3 cfu g^{-1} to between log 6 and log 7 cfu g^{-1} and

have an important impact on cheese quality (Beresford & Williams, 2004; Gobbetti, De Angelis, Di Cagno, Mancini, & Fox, 2015). Since *P. acidilactici* FAM18098 reached similar population densities in the semi-hard cheese, this study argues that this strain, and probably other dairy strains, is well adapted to the cheese environment. It is likely to provoke detectable biochemical processes during cheese ripening, as described in the following sections.

3.2. Lactate, citrate, fat, and short-chain fatty acids

The SCFA composition analysis (Table 1) revealed that the hard cheeses produced with *P. acidilactici* contained significantly less formate, propionate, butyrate, isovalerate, and hexanoate than the control cheeses. This finding was particularly pronounced in the hard cheeses. Supplementary material Table S1 shows the individual values to reproduce the statistical results. *P. acidilactici* FAM18098 either inhibited the formation of these compounds or metabolised them.

Interestingly, considerably higher amounts of acetate were found in all the cheeses made with *P. acidilactici* compared with their corresponding control cheeses. This can be explained by several metabolic activities that are associated with the formation of acetate. First, it could be derived from lactate, as reported for the closely related species *P. pentosaceus* (Thomas et al., 1985). However, this metabolic activity requires oxygen. The samples in this study were taken from the inside of the cheeses, which is an anaerobic environment, and were analysed shortly after sampling. No significant differences in the lactate content between control cheeses and the cheeses with *P. acidilactici* were measured (Table 1); therefore, it is unlikely that an oxidation of lactate to acetate did occur.

Second, acetate can be formed as a product during citrate degradation, which is a metabolic feature of various lactic acid bacteria (Beresford, 2011). No significant differences in the content of citrate between the control cheeses and the cheeses produced with *P. acidilactici* were determined. The inability of *P. acidilactici* FAM18098 and the starter cultures to ferment citrate was confirmed using agar plates that contained insoluble calcium citrate. No clear zones around the colonies were observed (data not shown). These findings demonstrated that citrate was not a precursor for acetate formation.

Third, acetate could also be the result of lipolysis; this study did not measure an influence on the fat content (Table 1). Beyond that, no clear evidence for lipid degradation capabilities can be taken from the scientific literature. Bhowmik and Marth (1989) detected esterase activity in the *P. pentosaceus* strains but not in the *P. acidilactici* strains. Litopoulou-Tzanetaki et al. (1989) did not detect lipolytic activity of *P. acidilactici* strains using tributyrin agar. Consequently, fat degradation probably did not considerably contribute to acetate formation.

Fourth, acetate could be derived through the heterofermentative fermentation of carbohydrates. The main carbohydrate source was lactose, which was present in the milk. However, in the cheese types produced in this study, the starter cultures completely metabolised lactose via the homofermentative pathway during the first 24 h of cheese making. Therefore, lactose cannot be the reason for the increased acetate formation.

In summary, the increased acetate levels present in the cheeses with *P. acidilactici* cannot be explained at the moment on the basis of the performed analyses. It is possible that the degradation of SCFA as well as the catabolism of amino acids played an important role. Thus, for example, the amino acids alanine, serine, cysteine, glycine, threonine, and tryptophan can be degraded to pyruvate, which could subsequently be metabolised to acetate by the combined action of pyruvate dehydrogenase (PDH), phosphotransacetylasae (Pta), and acetate kinase (Fig. 1). The genes encoding these enzymes are present in the *P. acidilactici* genomes deposited at the GenBank database (data not shown).

3.3. Proteolysis

Primary and secondary proteolysis were assessed by determining the NPN and TCA-N in relation to the TN; no significant differences were found (Table 2). This was confirmed by the sums of the free amino acids amounts obtained from the HPLC analysis (Table 3), which did not show significant differences between the cheeses. This indicates that the presence of the *P. acidilactici* strain did not contribute to protein degradation during cheese ripening. This is in line with studies performed by Litopoulou-Tzanetaki et al. (1989) and Bhowmik and Marth (1990), who found that *Pediococcus* spp., if present, only exhibited weak proteolytic activities.

3.4. Amino acid metabolism

The free amino acid composition analysis revealed significant differences between the control cheeses and the cheeses made with the *P. acidilactici* FAM18098. The cheeses made with *P. acidilactici* contained more ornithine, alanine, and AABA and less citrulline, serine, and threonine when compared with the control cheeses (Table 3). The nonproteinogenic amino acids AABA and ornithine are not present in the caseins and were therefore synthesised by *P. acidilactici*. Since there was no evidence that *P. acidilactici* enhanced proteolysis, it can be assumed that the species also produced alanine.

Ornithine can be synthesised via the arginine deiminase pathway. This pathway requires an amino acid permease, which imports arginine and exports ornithine, and the enzymes arginine deiminase, ornithine carbamoyltransferase, and carbamate kinase. The key enzyme arginine deiminase can be found in 32 *P. acidilactici* genomes deposited in the GenBank database. Additionally, arginine

Table 1
Lactic acid, citrate, fat, and short chain fatty acids in the cheeses at the end of ripening.[a]

Cheese variant	Adjunct	DMS	LMS	Citrate	C1	C2	C3	C4	iC4	iC5	C6	iC6	FDM
Hard, 120 d	No	67.5	46.0	8.6	7.7	61.2	7.7	18.4	1.2	0.9	1.3	1.7	514.3
Hard, 120 d	Yes	65.0	40.5	8.4	1.2	92.1	1.2	5.1	0.1	0.1	0.2	0.1	511.9
Semi-hard, 120 d	No	36.0	33.5	7.2	0.5	4.7	1.2	1.4	0.4	0.2	0.1	0.4	489.8
Semi-hard, 120 d	Yes	31.0	30.5	7.9	0.4	8.9	1.2	1.5	0.4	0.2	0.1	0.4	497.6
Semi-hard, 90 d	No	60.0	49.5	5.5	0.4	4.3	1.0	0.8	0.2	0.2	0.0	0.0	502.9
Semi-hard, 90 d	Yes	57.0	49.0	5.6	0.3	9.7	0.8	0.9	0.2	0.1	0.1	0.1	499.5
GLM	Adjunct	ns	ns	ns	***	***	***	***	ns	*	*	ns	ns

[a] Abbreviations are: DMS, D-lactate; LMS, L-lactate; C1, formate; C2, acetate; C3, propionate; C4, butyrate; C5, valerate; C6, caproate; iC4, isobutyrate; iC5, isovalerate; iC6, isocaproate; FDM, fat in dry matter. Results (in mmol kg^{-1} except FDM in g kg^{-1}) are the means of two cheese making experiments. Asterisks indicate a significant difference (*$P < 0.05$, ***$P < 0.001$; general linear model) between control cheeses and cheeses made with the pediococcal adjunct; ns, not significant.

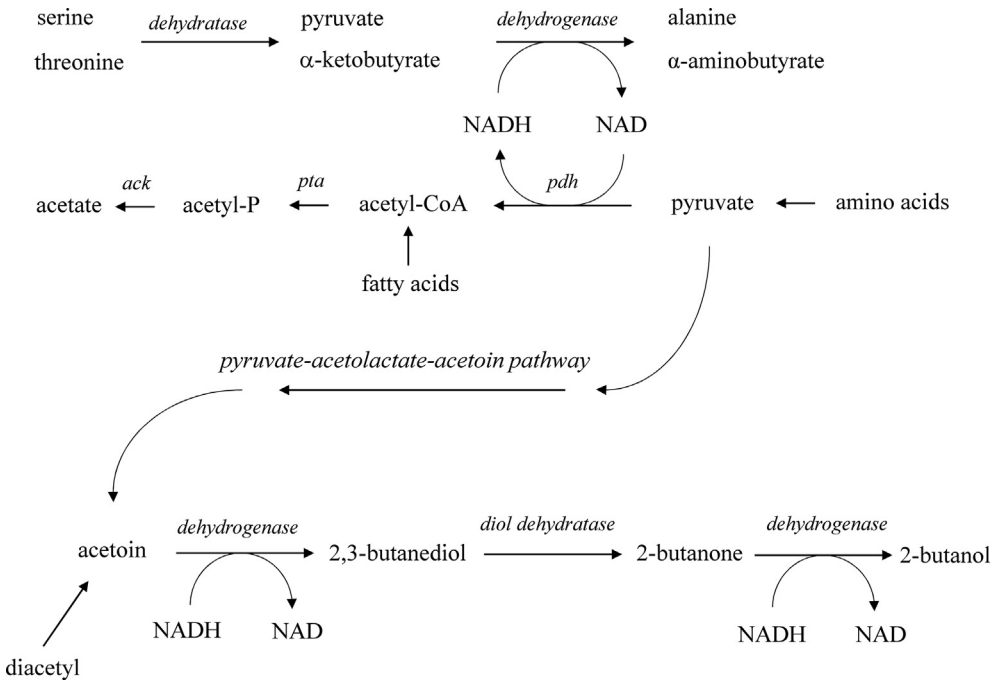

Fig. 1. Schematic illustration of the putative pathways leading to the formation of α-aminobutyrate, alanine, acetate, and 2-butanol in the cheese in the presence of *P. acidilactici* FAM18098. Abbreviations are: pdh, pyruvate dehydrogenase; pta, phosphotransacetylase; ack, acetate kinase; NAD, nicotinamide adenine dinucleotide (oxidised form); NADH, nicotinamide adenine dinucleotide (reduced form).

Table 2
Moisture, total nitrogen (TN), water-soluble nitrogen (WSN), and TCA-soluble nitrogen (TCA-N) in cheese after ripening.[a]

Cheese variant	Adjunct	Moisture	TN	WSN	TCA-N	TCA-N/TN	WSN/TN
Hard, 120 d	No	335.8	42.8	12.2	8.1	18.9	28.6
Hard, 120 d	Yes	333.5	43.2	12.2	7.8	18.1	28.2
Semi-hard, 120 d	No	387.3	41.4	13.4	7.0	16.8	32.3
Semi-hard, 120 d	Yes	378.5	41.5	13.2	7.1	17.0	31.7
Semi-hard, 90 d	No	447.5	40.1	12.6	6.6	16.5	31.4
Semi-hard, 90 d	Yes	455.5	39.7	12.7	6.8	17.2	32.1

[a] Results (in g kg^{-1} for moisture, TN, WSN and TCA-N) are the means of two cheese-making experiments; no significant differences were found between results with and without adjunct.

hydrolysis has been recorded for several strains of *P. acidilactici* (Holzapfel et al., 2006). The *P. acidilactici* strain used in this study produced ornithine when incubated in the arginine-containing medium MAM. Thus, the presence of an arginine deiminase pathway is a species-specific property of *P. acidilactici*. Since, besides ornithine, ammonia, adenosine triphosphate, and carbon dioxide are produced by this pathway, the presence of this species in cheese could influence pH and be causative for eye formation. The formation and accumulation of putrescine could occur in interaction with ornithine decarboxylase-positive bacteria.

Nonproteinogenic ornithine was also found in the control cheeses, but at significantly lower levels than in the cheeses produced with the pediococci. This is related to the starter cultures used in this study. It was found that RMK 124 and MK 401 also produced ornithine when they were incubated in the MAM medium (data not shown). This can be explained by the presence of *Lb. delbrueckii* subsp. *lactis* strains, which have arginine deiminase activity (Wenzel et al., 2018); in MK 401, *Lactobacillus lactis* strains could also play a role. Researchers found that this species also possessed the arginine deiminase pathway (Chou, Weimer, & Cutler, 2001). Nevertheless, it is striking that the control cheeses contained more citrulline than the cheeses made with *P. acidilactici*. This suggests that the starter cultures convert citrulline to ornithine at a very low rate. This effect was also observed with other thermophilic starter cultures that contained *Lb. delbrueckii* subsp. *lactis* strains exhibiting arginine deiminase activity (Wenzel et al., 2018).

The higher levels of AABA and alanine together with the lower levels of serine and threonine in the cheeses with *P. acidilactici* FAM 18098 are remarkable. A similar observation was made when this strain was cultivated in serine-threonine-containing broth (Irmler et al., 2013). It suggests that serine and threonine are used as precursors for alanine and AABA biosynthesis. This is supported by the mathematical calculation in which the amounts of alanine, AABA, serine, and threonine are summed and divided by the total amount of free amino acids. Equal values are obtained from this calculation for the control cheeses and their corresponding cheeses made with *P. acidilactici* FAM18098 (data not shown).

AABA biosynthesis can be found in various strains of this species (Irmler, unpublished). It has, to the best of the present researchers' knowledge, not been reported for a lactic acid bacterium thus far. The metabolic pathway probably involves a threonine dehydratase (EC 4.3.1.19) that deaminates threonine and serine to α-ketobutyrate and pyruvate, respectively (Fig. 1). These compounds are then converted to α-aminobutyrate and alanine, respectively. The latter step could be catalysed by a dehydrogenase or an aminotransferase. Interestingly, two neighbouring genes that encode a threonine

Table 3
Free amino acids in the cheeses after ripening.[a]

Cheese variant	Adjunct	Ala	AABA	Arg	Asn	Asp	Cit	GABA	Gln	Glu	Gly	His	Ile	Leu	Lys	Met	Orn	Phe	Pro	Ser	Thr	Trp	Tyr	Val	FAS
Hard, 120 d	No	8.3	0.0	0.2	12.8	4.7	5.7	0.4	10.2	38.2	8.0	8.1	11.6	24.7	27.4	6.8	4.0	12.0	23.9	9.1	9.9	1.0	6.0	19.7	252.5
Hard, 120 d	Yes	16.2	9.7	0.1	12.5	4.6	0.3	0.1	10.1	39.6	8.0	7.6	10.9	23.8	25.3	6.5	8.3	11.1	23.1	0.5	0.3	0.9	5.5	19.1	244.0
Semi-hard, 120 d	No	5.6	0.0	0.3	8.8	2.7	3.8	1.4	6.0	21.6	4.6	4.8	5.9	19.4	24.1	4.0	6.1	8.8	12.5	4.4	4.6	0.4	4.2	12.8	166.6
Semi-hard, 120 d	Yes	10.7	5.5	0.0	9.2	3.0	0.2	1.4	6.4	23.8	4.8	5.2	6.5	19.9	26.7	4.4	12.8	8.7	13.3	0.4	0.3	0.4	4.4	13.8	181.6
Semi-hard, 90 d	No	6.2	0.0	0.1	9.6	2.7	2.3	3.5	7.9	17.9	4.8	4.2	6.6	22.4	25.8	4.3	12.5	9.5	11.2	5.0	4.8	0.3	4.8	13.8	179.9
Semi-hard, 90 d	Yes	13.9	5.8	0.0	11.2	3.2	0.0	4.2	9.6	22.5	6.2	5.8	8.2	25.7	34.9	5.0	31.8	10.6	13.4	0.7	0.2	0.4	5.3	16.5	235.1
GLM	Adjunct	***	***	***	ns	ns	***	ns	ns	ns	ns	ns	ns	ns	ns	ns	***	ns	ns	***	***	ns	ns	ns	ns

[a] The results (mmol kg^{-1}) are the means of two cheese-making experiments; asterisks indicate a significant difference (***$P < 0.001$; general linear model; ns, not significant) between control cheese and cheese made with the pediococcal adjunct.

Table 4
Volatile organic compounds in the cheeses after 90 days of ripening.[a]

Cheese variant	Adjunct	Diacetyl	2-Pentanone	3-Methyl-butanal	2-Heptanone	2-Nonanone	2-Undecanone	2-Butanone	2-Butanol (total ion counts)
Hard, 120 d	No	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0
Hard, 120 d	Yes	0.63	0.52	0.75	0.97	1.04	1.12	49.61	520510
Semi-hard, 120 d	No	1.00	1.00	1.00	1.00	1.00	nd	1.00	0
Semi-hard, 120 d	Yes	0.46	1.09	1.24	1.16	1.06	nd	81.37	13421262
Semi-hard, 90 d	No	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0
Semi-hard, 90 d	Yes	0.45	0.91	1.58	1.13	1.25	0.95	5.13	2840557
GLM	Adjunct	*	ns	ns	ns	ns	ns	ns	na

[a] For statistical reasons, the peak areas for each compound except 2-butanol were transformed into relative values, with the control cheese set as 1. The results are the means of two cheese-making experiments (nd, not detected). An asterisk indicates a significant difference (*$P < 0.05$; general linear model; ns, not significant, na, not applied) between control cheese and cheese made with the pediococcal adjunct.

dehydratase (*HMPREF0623_RS00370*) and an alanine dehydrogenase (*HMPREF0623_RS00365*) are present in the *P. acidilactici* DSM 20284 genome. Threonine dehydratases are divided into two classes: catabolic threonine dehydratase and biosynthetic threonine dehydratase. The latter is involved in isoleucine biosynthesis (Yu, Li, & Wang, 2013). An extensive study that compared the protein sequences of threonine dehydratases predicted that the *P. acidilactici* enzyme belonged to the catabolic threonine dehydratases and not to the biosynthetic threonine dehydratases (Yu et al., 2013). This prediction needs validation by analysing the enzymatic properties of the threonine dehydratase of *P. acidilactici*.

With regard to alanine dehydrogenase, Hols et al. (1999) showed that the introduction of an alanine dehydrogenase-encoding gene from *Bacillus sphaericus* into *Lactobacillus lactis* enabled the genetically modified strain to produce alanine from pyruvate. Analogically, the alanine dehydrogenase from *P. acidilactici* could be involved in alanine formation. The alanine dehydrogenase could use not only pyruvate but also α-ketobutyrate, which would result in AABA synthesis as it was demonstrated for an alanine dehydrogenase from *B. sphaericus* (Ohashima & Soda, 1979).

Dehydrogenases catalyse oxidation and reduction reactions and often use nicotinamide adenine dinucleotide (NAD) as electron acceptors. This study argues that alanine and AABA are used as electron sinks to produce NAD, which *P. acidilactici* uses to generate energy (e.g., via the acetate kinase-phosphotransacetylase pathway) (Fig. 1).

3.5. Volatile organic compounds

The formation of VOCs is often associated with the presence of nonstarter lactic acid bacteria (Gobbetti et al., 2015). When the volatile profile of the ripened cheeses was analysed using HS-SPME-GC-MS, the levels of diacetyl were significantly lower, whereas the levels of 2-butanol were higher in the cheeses manufactured with *P. acidilactici* FAM18098 than in the cheeses that did not contain this strain (Table 4). Furthermore, the cheeses with *P. acidilactici* clearly tended to have higher levels of 2-butanone than their corresponding control cheeses. Guarrasi et al. (2017) also observed that *P. acidilactici* produced 2-butanol when incubated in a cheese-based medium. One possible metabolic pathway could involve the formation of α-acetolactate from pyruvate. The former compound can then be converted to 2-butanol via acetoin; 2,3-butanediol; and 2-butanone (Fig. 1). This pathway is present in several bacteria, including lactic acid bacteria (Celińska & Grajek, 2009). For example, the formation of 2-butanol from 2,3-butanediol via the intermediate 2-butanone has been found in strains of *Lactobacillus fermentum*, *Lactobacillus buchneri*, and *Lactobacillus brevis* (Ghiaci, Lameiras, Norbeck, & Larsson, 2014; Speranza et al., 1997). As proposed for alanine and AABA, 2-butanol could be a sink for electrons (Fig. 1); however, whether a metabolic route from pyruvate to 2-butanol exists in *P. acidilactici* requires further investigation.

4. Conclusions

Although *P. acidilactici* occurs in ripened cheese, there is limited knowledge about its metabolic activities within the cheese. The present study showed that *P. acidilactici* FAM18098 possesses biochemical activities that considerably impacted cheese ripening and quality. Due to the presence of the arginine deiminase pathway, arginine was converted to ornithine. Furthermore, *P. acidilactici* FAM18098 catabolised serine and threonine while concomitantly forming α-aminobutyrate and alanine. Additionally, that strain had formed 2-butanol and more acetate in the cheese than the control cheeses made without this strain. It is possible that these metabolic activities are linked to the formation of carbon dioxide, as is the case for the arginine deiminase pathways. The use of isotope-labelled compounds could help researchers to gain deeper insight into the interconnections of the metabolic pathways of this species.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2019.04.001.

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